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# Chemical detoxification vs mechanical removal of host plant toxins in *Eucalyptus* feeding sawfly larvae (Hymenoptera: Pergidae)

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ABSTRACT

The essential oils that characterize the eucalypts and related Myrtaceae pose a challenge for herbivores. Phytophagous insects that feed on oil-rich Myrtaceae have developed specific mechanisms to deal with these oils, some of which are notoriously toxic (e.g. 1,8-cineole). Some of the eight Australian subfamilies in the sawfly family Pergidae are associated exclusively with *Eucalyptus* and *Melaleuca* species that often have high concentrations of essential oils. Unexpectedly, the Perginae and Pterygophorinae use different mechanisms to deal with the same toxic components in their respective host plants. Larvae of the Perginae have the inner surface of their mandibles equipped with soft brush-like structures that are unique among phytophagous insects in general. The proposed role of these ancillary mandibular structures in separating leaf oils from nutritive plant matter could be confirmed in experiments with larvae of two pergine species. The oil sequestration is, however, incomplete and chemical gut content analyses by gas-chromatography (GC) revealed that 1,8-cineole does enter the midgut and is metabolised to hydroxycineole. Although the related Pterygophorinae also feed mainly on oil-rich Myrtaceae, they do not sequester the oil and lack morphological structures on their mandibles. Chemical analysis of the gut content of two pterygophorine species showed that they rely solely on chemical detoxification of the relevant plant compounds, with GC demonstrating that the 1,8-cineole is removed far more rapidly and completely than in the pergine species.



## 1. Introduction

A characteristic feature of eucalypts and some related Myrtaceae is their high content of secondary plant compounds (Boland et al., 1991) and some of these are toxic, even lethally so, to insects without specific defence mechanisms (Gershenzon and Croteau, 1992; Mabry and Gill, 1992). The insects that feed habitually on these plants and ingest their toxic secondary compounds often have specific mechanisms to deal with them, as reviewed by Cooper (2001). In particular, they deal with the toxic terpenoids that predominate in the eucalypts and related oil-rich Myrtaceae in four main ways. Some insects avoid the oil glands while feeding (e.g., the jarrah leafminer *Perthida glyphopa* Common (Mazanec, 1983), neonate and early instar larvae of *Mnesampela private* (Steinbauer and Matsuki, 2004)), some apparently tolerate most oils that pass through their digestive system (e.g., the chrysomelid beetle *Paropsis atomaria* Olivier (Morrow and Fox, 1980)), some remove terpenoids by sequestering oils/terpenoids in sac-like extensions of the foregut (e.g., some pergid sawflies (Morrow et al., 1976; Tait, 1962)), or they detoxify ingested terpenoids by converting them to other compounds, as in some chrysomelid beetles and weevils (Ohmart and Larsson, 1989; Southwell et al., 1995).

A few species are known to combine more than one of these methods. Some myrtaceous feeding pergid sawflies, for example, sequester terpenoids into their diverticulum as well as detoxify any terpenoids that nevertheless do enter the midgut (Schmidt et al., 2000). These pergids belong to the subfamily Perginae, one of three Australian pergid subfamilies (the others are the Pterygophorinae and Phylacteophaginae) that radiated almost exclusively on myrtaceous hosts and thus are exposed to high concentrations of essential oils in their host plants. A preliminary phylogenetic analysis of the family Pergidae, which has 14 subfamilies and with eight of them occurring in Australia, places the Myrtaceae associated subfamilies in separate lineages from one another (Schmidt et al., 2006). In this study we focus on two subfamilies, Perginae and Pterygophorinae, with morphologically and behaviourally very different types of larvae that apparently developed radically different mechanisms to deal with the toxic compounds that occur in their myrtaceous host plants.

The major and most toxic component of the leaf oils in many *Eucalyptus* species and plants in related genera is the monoterpene

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1,8-cineole (Boland et al., 1991). It serves a protective function against herbivorous mammals like possums (McLean et al., 2007), and has even been investigated as a contact and fumigant toxin against stored grain pests (Abeywickrama et al., 2006; Lee et al., 2001; Rozman et al., 2007; Tripathi et al., 2001). Unlike most other terpenoids, which evidently pass through the gut of herbivorous insects unchanged (Southwell et al., 1995, 2003), cineole is selectively metabolised and converted into a range of non-toxic forms in various ways by insects from different taxonomic orders (Ohmart and Larsson, 1989; Schmidt et al., 2000; Southwell et al., 1995, 2003).

Larvae of the subfamily Perginae have a soft brush-like structure on the inner surface of their mandibles that is unique among phytophagous insects, and which is postulated to be central in separating oils from the leaf material they ingest (Schmidt et al., 2000). The oil is subsequently stored in pharyngeal diverticula without chemical modification (Morrow et al., 1976; Schmidt et al., 2000) and the larvae, commonly called spitfire grubs, use the oil for defence when disturbed (Bennet and Scott, 1859; Froggatt, 1901; Westwood, 1880). The original (or primary) function of the extraction of leaf oils was possibly to reduce the toxicity of ingested plant tissue, because larvae of *Pergagrapta polita* (Leach) periodically void some of the content of the diverticula without being disturbed, presumably when the diverticula are full (Schmidt et al., 2000). Here we test aspects of the postulated operation of the oil extraction mechanism in larvae of *P. polita*. Initially, we quantified the oil extraction and storage dynamics. Specifically, the volume of leaf oil taken in daily by the larvae was assessed by measuring their leaf consumption. We then investigated, with ablation techniques, the proposed role of the mandibular brushes in the extraction of essential oils from the leaf material as it is ingested.

Although the Pterygophorinae are also associated with oil-rich Myrtaceae, they lack the morphological features that Perginae use to extract oil from ingested leaf material, and presumably have to rely solely on enzymatic detoxification of the 1,8-cineole in their diet. If oil separation and storage in the Perginae evolved not only for defence against predators, but primarily to eliminate toxic leaf oils from the diet (Schmidt et al., 2000), we would expect sawfly larvae in the subfamily Perginae to have much lower levels of enzyme based detoxification of cineole than do sawfly larvae in the subfamily Pterygophorinae.

To investigate the detoxification mechanisms of the two subfamilies, the metabolism of 1,8-cineole was examined in two pergine species (*P. polita* and *Pergagrapta* sp.) and two from the subfamily Pterygophorinae (*Lophyrotoma interrupta* (Klug) and *Pterygophorus insignis* Kirby). This allowed us to compare the efficiency and mode of the enzymatic detoxification of cineole between species of the two sawfly subfamilies.

## 2. Materials and methods

### 2.1. Leaf oil discharge by P. polita larvae

To quantify the amount of oil that is emitted by a larva during a defence reaction, actively feeding mature larvae (5th and 6th instars) of *P. polita* from several clusters collected in the field near Brisbane were anaesthetized using $CO_2$ and randomly assigned to two groups of 20 larvae each. All larvae were weighed individually and then harassed every 10–15 s by touching them gently with a cotton tipped applicator. The oil exuded from the mouth of each treatment larva was absorbed into the cotton wool to remove it, whereas the larvae of the other group were allowed to re-ingest the liquid into their diverticula, which they did readily. The harassment was stopped when the larva showed a noticeable decrease in responsiveness, usually within 3–5 min. Because larvae usually feed in close association, a third group of 20 larvae was established of which each larva was treated in the same way as larvae of the control group, i.e. they were allowed to re-ingest the oil. The group of larvae was used in the group feeding treatment described below.

### 2.2. Leaf consumption of P. polita larvae

To examine if leaf consumption is different between larvae with filled diverticula and ones with a reduced diverticular content, larvae of the three groups described in the previous section were placed in containers (15 cm × 6.5 cm diam), one larva per container in the solitary feeding treatment and solitary feeding control, and four per container in the group feeding control. One freshly picked twig of *Melaleuca quinquenervia*, with 3–5 mature leaves, was placed in each container that contained a solitary larva, after the entire leaf area had been measured by scanning with a flatbed scanner. Each container of group feeding larvae received two such twigs. The leaves were removed and replaced daily with new ones during the day time when larvae do not feed. Each day of the experiment, the area of each old leaf was measured to quantify leaf consumption by comparing the area before and after feeding. The frass was weighed and the number of pellets recorded. For the group feeding larvae, frass weights were pooled and that was divided by four, as was the number of pellets.

To assess the relationships between *M. quinquenervia* leaf length, width, length × width, leaf area, and leaf weights, 100 mature fresh leaves were selected randomly, weighed and scanned. Leaves were taken from trees in the same area in which larvae had been collected for the experiment. Leaf length, width, and area were inferred from scanned images using the ImageJ software package (Rasband, 1997–2008). Leaf area was measured directly using the "Analyze Particles" function of the program, and inferred indirectly by measuring and multiplying leaf length and width. Leaf measurements showed a strong and highly significant correlation with leaf weight, with all correlations being >0.90. The weight of the leaf area eaten by the larvae was calculated from the linear regression formula: leaf weight = $-12.39 + 47.80 \times$ leaf area.

### 2.3. Ablation of the mandibular brush

To test the role of the mandibular brush in separating leaf oils from leaf material, 20 actively feeding mature larvae (5th and 6th instars) of *P. polita* were randomly assigned to one of two groups. All larvae were of the same age and from the same field-collected larval cluster. Examination of smaller (male) and larger (female) larvae from the same cluster did not exhibit any differences of mandibular brush structures between sexes. Each larva was anaesthetized using $CO_2$ and those in the treatment group had the mandibular brushes of both mandibles removed using fine forceps. The brush is only narrowly attached to the mandible (Schmidt et al., 2000) and is readily detached in this way. The larvae were then placed in containers (15 cm × 6.5 cm diam), two from the same treatment/control group per container, and kept in the laboratory at room temperature with 16/8 h day/night. Two twigs of *M. quinquenervia* with 3–5 leaves each were placed in each container and larvae were allowed to feed overnight. The following day all larvae were euthenased and dissected so that food samples could be taken from the foregut and midgut and transferred immediately into n-hexane. Larvae have an empty foregut in the evening, when they start feeding, and those that still had an empty foregut in the morning evidently had not fed and were omitted from further consideration. In a few cases the diverticulum was damaged during dissection, and contamination of gut samples with concentrated oil from the diverticulum was possible. Those larvae were therefore excluded from analysis.